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Mutations converting cyclodextrin glycosyltransferase from a transglycosylase into a starch hydrolase

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Abstract Cyclodextrin glycosyltransferase (CGTase) efficiently catalyzes transglycosylation of oligo-maltodextrins, although the enzyme also has a low hydrolytic activity. Its +2 substrate binding subsite, which contains the conserved Phe184 and Phe260 residues, has been shown to be important for this transglycosylation activity [Nakamura et al. (1994) *Biochemistry* 33, 9929–9936]. Here we show that the amino acid side chain at position 260 also controls the hydrolytic activity of CGTase. Three Phe260 mutants of *Thermoanaerobacterium thermosulfurigenes* CGTase were obtained with a higher hydrolytic activity than ever observed before for a CGTase. These Phe260 mutations even changed CGTase from a transglycosylase into a starch hydrolase. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Cyclodextrin glycosyltransferase; Transglycosylation; Transglycosidase; Enhanced hydrolysis; Acceptor subsite

1. Introduction

The α -amylase family, or glycoside hydrolase family 13, is a large family of starch processing enzymes [1,2]. The catalytic site residues and the α -retaining bond cleavage mechanism are strictly conserved in all members of this family [3,4], but the product and reaction specificity vary widely.

Cyclodextrin glycosyltransferase (CGTase) (EC 2.4.1.19) is also a member of this family. It forms circular α -(1,4)-linked oligosaccharides (cyclodextrins) from linear α -(1,4)-linked oligosaccharide substrates. This reaction proceeds via a covalent intermediate [4,5]. The non-reducing end of this intermediate is subsequently used as the acceptor that cleaves the covalent enzyme–substrate bond, and a cyclodextrin is released. CGTase can also use water or the non-reducing end of a free oligosaccharide as acceptor, which results in hydrolysis or a disproportionation reaction, respectively.

Whereas α -amylase is a strongly hydrolytic enzyme,

CGTase is first of all a transglycosylase. The hydrolysis activity of CGTases is in general much lower than the disproportionation and cyclization activities. Only the CGTases from *Thermoanaerobacter* [6] and *Thermoanaerobacterium thermosulfurigenes* strain EM1 (*Tabium*) [7] have relatively high hydrolysis activities, although still very low compared to α -amylases. The ratio between hydrolysis and transglycosylation is determined by the nature of the acceptor used and by the properties of the CGTase acceptor subsites. While acceptor subsite +1 is conserved in CGTases and α -amylases, the +2 subsite is only conserved in CGTase [2,8–15]. At this subsite Phe184 and Phe260 interact with bound oligosaccharides (Fig. 1). Furthermore, it has been suggested that binding of sugars at the acceptor subsites, but not water, activates CGTases for catalysis [16]. This induced-fit model can explain the transglycosylation specificity of CGTase with its associated low hydrolysis activity.

Here we describe mutations of three aromatic residues in *Tabium* CGTase. Phe184 and Phe260 are part of subsite +2, while Phe196 is located in the center of the active site cleft (Fig. 1). All three residues interact with substrates and mutagenesis experiments have already demonstrated the importance of these residues in the transglycosylation activities of CGTase [8,17–22]. Mutation of Phe260 strongly enhanced the hydrolysis activity, converting CGTase into an enzyme preferring hydrolysis to transglycosylation.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Escherichia coli DH5 α [23] was used for DNA manipulations. Plasmid pCT2, with the *Tabium* *cgt* gene [24], was used for site-directed mutagenesis and sequencing. CGTase proteins were produced with plasmid pCSgt-tt [25] and *Bacillus subtilis* strain DB104A as host [26]. Plasmid carrying strains were grown on LB medium [27] at 37°C in the presence of ampicillin (100 μ g/ml) or kanamycin (50 μ g/ml for *E. coli* and 5 μ g/ml for *B. subtilis*).

2.2. Production and purification of CGTase proteins

CGTase (mutant) proteins were produced and purified as described [28]. Purity and molecular weight were checked by SDS–polyacrylamide gel electrophoresis. Enzyme concentrations were determined using the Bradford reagent from Bio-Rad (Munich, Germany) and bovine serum albumin as standard.

2.3. DNA manipulations

Mutants were constructed in pCT2 as described earlier and verified by DNA sequencing [13]. Mutations were subsequently introduced in pCSgt-tt by exchanging its 1300 bp *ScaI/DraIII* fragment for the

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Abbreviations: CGTase, cyclodextrin glycosyltransferase; *Tabium*, *Thermoanaerobacterium thermosulfurigenes* strain EM1; BC251, *Bacillus circulans* strain 251; EPS, 4-nitrophenyl- α -D-maltoheptaoside-4,6-O-ethylidene

corresponding fragment of pCT2 carrying the mutation. The following oligonucleotides were used: F184S, 5'-CATTATGGAGGTACCGATTCTTCATCTTATG-3' (*Kpn*I); F196G, 5'-TATCGTAACCTTAGGTGGTGAATTTAGCAGATCTAAATCAAC-3' (*Bgl*II); F260L, 5'-CCAGTATTTACATTTGGAGAGTGGTACTTGGAAACG-3'; F260N, 5'-CCAGTATTTACATTTGGAGAGTGGAAATCTTGGAAACG-3'; F260H, 5'-CCAGTATTTACATTTGGAGAGTGGCATCTTGGAAACG-3'; F260R, 5'-CCAGTATTTACATTTGGAGAGTGGCGTCTTGGAAACG-3'; F260E, 5'-CCAGTATTTACATTTGGAGAGTGGGAACTTGGAAACG-3'; F260I, 5'-CCAGTATTTACATTTGGAGAGTGGATTCTTGGAAACG-3'. Restriction sites are underlined. Mutations in Phe260 resulted in the loss of a *Stu*I restriction site. The double mutants F184S/F260N and F196G/F260N were made using plasmid pCT2 with the F260N mutation as template in the PCR reactions.

2.4. Enzyme assays

All enzyme assays were performed in 10 mM sodium citrate buffer (pH 6.0) at 60° C.

2.5. β -Cyclodextrin forming activity

β -Cyclodextrin forming activity was determined by incubating 0.1–5.0 μ g enzyme/ml with a 2.5% (w/v) solution of partially hydrolyzed potato starch with an average degree of polymerization of 50 (Paselli SA2; Avebe, Foxhol, The Netherlands). The amount of β -cyclodextrin produced was quantified with phenolphthalein [29]. One unit of activity is defined as the amount of enzyme producing 1 μ mol of β -cyclodextrin per min.

2.6. Disproportionation activity

Disproportionation activity was determined as described before [30,31], using 0.1–5.0 μ g enzyme/ml, 1 mM 4-nitrophenyl- α -D-maltoheptaoside-4,6-*O*-ethylidene (EPS; Roche) and 10 mM maltose as donor and acceptor substrates, respectively. One unit of activity is defined as the amount of enzyme converting 1 μ mol of EPS per min.

2.7. Hydrolyzing activity

Hydrolyzing activity was measured by following the increase in reducing power, using 1% (w/v) soluble starch (Lamers and Pleuger, Belgium) and 1 μ g/ml of enzyme [20]. One unit of activity is defined as the amount of enzyme producing 1 μ mol of reducing sugars per min.

2.8. Formation of cyclodextrins and linear products

Formation of cyclodextrins and linear products from 10% (w/w) pregelatinized starch (Paselli WA4; Avebe) was measured by incubating the starch solution for 24 h with 0.25 U/ml β -cyclodextrin forming

activity (0.05 U/ml for F260R and F196G/F260N). Products formed were analyzed by a high pressure liquid chromatograph equipped with an Econosphere NH2 5U column (250 by 4.6 mm; Alltech Nederland, Breda, The Netherlands) eluted with acetonitrile/water (60/40, v/v; 1 ml/min).

3. Results and discussion

3.1. Phenylalanine residues 184, 196 and 260 are important for transglycosylation activity

To investigate the importance of subsite +2 and residue 196 for the various activities of CGTase we mutated three conserved phenylalanines (Phe184, Phe196 and Phe260 in *Tabium* CGTase). The strongly reduced cyclization and disproportionation activities of the Phe184, Phe196 and Phe260 mutants (Table 1) are in agreement with results of mutations of the corresponding residues in other CGTases [8,17–21]. The double mutants F184S/F260N and F196G/F260N showed even a stronger reduction in cyclization and disproportionation activity (Table 1) than would be expected from a combination of the single mutants suggesting that Phe184/Phe260 and Phe196/Phe260 act synergistically in these reactions. These results are consistent with a proposed induced-fit mechanism for CGTase, in which sugar binding at the acceptor subsites induces structural rearrangements that activate catalysis [16]. Thus, the decreased transglycosylation activities show that phenylalanine residues 184, 196 and 260 are important in the cyclization and disproportionation reactions.

3.2. Phe196 is important for transglycosylation and hydrolysis

The centrally located Phe196 residue (Fig. 1) has been suggested to play a role in limiting the hydrolysis activity of CGTase, since α -amylases (hydrolases) have a small residue (glycine, alanine, serine) at this position [14]. Indeed the Y195G mutation in BC251 CGTase, which is equivalent to position 196 in *Tabium* CGTase, shows an increased hydrolytic activity [20]. Unexpectedly, however, the corresponding F196G mutant of *Tabium* CGTase has a two-fold lower hy-

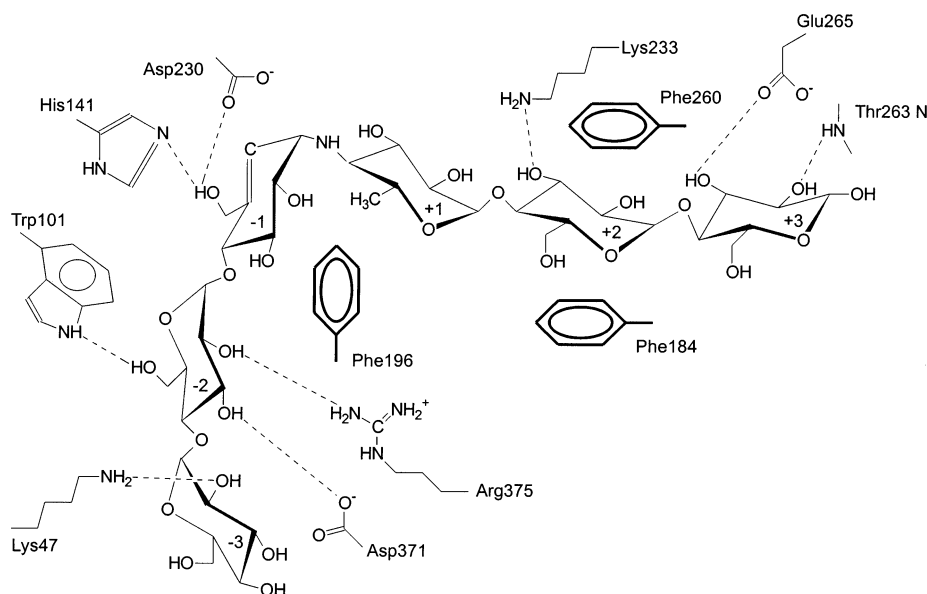


Fig. 1. Schematic overview of the interactions between *Tabium* CGTase and a maltohexaose inhibitor bound from subsites –3 to +3. For clarity, not all interactions at subsites –1 and +1 are shown [13].

Table 1
Hydrolysis, cyclization and disproportionation activities of *Tabium* wild-type and mutant CGTases at pH 6.0 and 60°C

Enzyme	Hydrolysis of starch ($\mu\text{mol}/\text{min}/\text{mg}$)	β -Cyclization ($\mu\text{mol}/\text{min}/\text{mg}$)	Disproportionation ($\mu\text{mol}/\text{min}/\text{mg}$)
Wild-type	54 \pm 4	240 \pm 9	510 \pm 23
F184S	60 \pm 7	52 \pm 6	230 \pm 12
F196G	30 \pm 3	27 \pm 2	36 \pm 2
F260N	117 \pm 12	31 \pm 3	95 \pm 7
F260L	174 \pm 10	33 \pm 2	167 \pm 10
F260I	174 \pm 7	17 \pm 1	218 \pm 5
F260E	177 \pm 13	19 \pm 1	21 \pm 4
F260H	25 \pm 3	74 \pm 3	177 \pm 14
F260R	17 \pm 2	0.7 \pm 0.2	< 1
F184S/F260N	118 \pm 11	5 \pm 1	20 \pm 2
F196G/F260N	15 \pm 1	0.8 \pm 0.2	< 1

hydrolytic activity (Table 1). Thus, Phe196 is important not only for the transglycosylation activity, but also for the enzyme's hydrolytic activity. Despite this, mutant F196G produced three times more linear products from starch than wild-type CGTase over a 24 h incubation period (Fig. 2). This indicates that residue Phe196 is a clear determinant for the linear versus circular product specificity of CGTase.

3.3. Phe260 limits the hydrolytic activity

Hydrolysis is a minor activity of CGTases. Nevertheless, several mutations are known to increase the enzyme's hydrolytic activity [19,20,32,33]. The most substantial enhancement of hydrolytic activity is, however, achieved by mutations of subsite +2 residues (Table 2). It has been suggested that the hydrophobicity of Phe183 and Phe259 at subsite +2 limits the hydrolytic activity of BC251 CGTase [19]. Mutation of the corresponding residues in *Tabium* CGTase showed that the F184S mutation changed the hydrolysis rate only slightly (Table 1). In contrast, mutations in Phe260 either significantly decreased (F260H,R) or increased (F260N,L,I,E) the hydrolytic activity (Table 1). Mutation F260N doubled the hydrolytic activity, whereas the corresponding F259N mutation in BC251 CGTase increased the hydrolytic activity 20-fold (Tables 1 and 2). Both mutants, however, enhanced the hydrolytic activity with about 60 units, suggesting that the muta-

tions have a similar effect in both CGTases and that the 20-fold enhancement is a consequence of the low hydrolytic activity of BC251 CGTase. The hydrolytic activity of CGTase thus is strongly modulated by the identity of residue 260. Furthermore, our mutations reveal that it is not the hydrophobicity of the side chain at position 260 that is important for the enzyme's hydrolytic, since similar-sized hydrophobic (F260I,L) as well as hydrophilic (F260N,E) substitutions increase the hydrolytic activity to a similar extent (Table 1). Therefore we conclude that the aromatic side chain of Phe260 is instrumental in limiting the hydrolytic activity of CGTase.

Until now wild-type *Thermoanaerobacter* CGTase displayed the highest hydrolytic activity [34]. Three *Tabium* mutants (F260L,I,E), however, possess even higher hydrolytic activities, and significantly lower cyclization activities (Table 1). The data show that single mutations can change CGTase reaction specificity from cyclization into hydrolysis. A product analysis after 24 h incubation of starch with (mutant) CGTases also showed that these Phe260 mutants produced substantially more linear than circular products (Fig. 2). Single mutations thus change CGTase product specificity from cyclodextrins into oligo-maltodextrins.

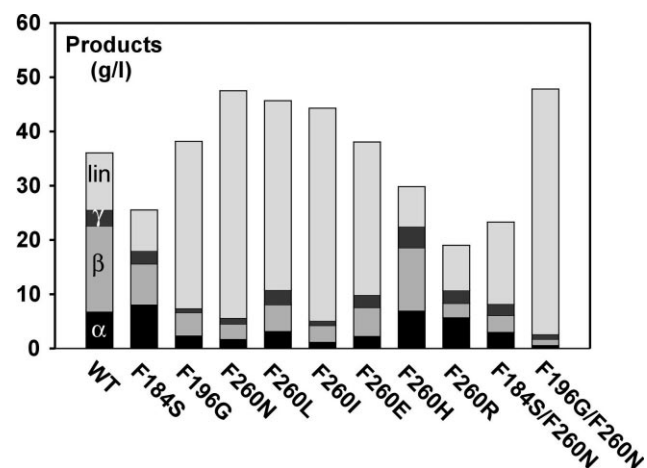


Fig. 2. Cyclodextrin and linear oligosaccharide (glucose to malto-octaose) products formed during incubation of the *Tabium* CGTase variants with 10% (w/v) starch for 24 h at 60°C. From bottom to top, α -CD (black), β -CD (gray), γ -CD (dark gray) and linear oligosaccharides (light gray).

Table 2
Subsite +2 mutant CGTases described in the literature and their hydrolysis activities

CGTase	Hydrolysis activity ($\mu\text{mol}/\text{min}/\text{mg}$)	Reference
<i>Tabium</i>		
Wild-type	54	this study
<i>B. circulans</i> strain 251		
Wild-type	3	[19]
F183S	9	[19]
F184N	11	[19]
F259S	33	[19]
F259N	60	[19]
<i>Bacillus stearothermophilus</i>		
Wild-type	2	[18]
F260I	4	[18]
<i>Bacillus</i> sp. 1011		
Wild-type	ND	[17]
F183L	ND	[17]
F259L	ND	[17]
<i>Thermoanaerobacter</i>		
Wild-type	82	[34]
Chemically modified	127	[34]

The *Thermoanaerobacter* CGTase is included, because the wild-type enzyme has a high hydrolytic activity. ND, not determined.

4. Conclusions

Our results show that Phe260 has an important role in limiting the hydrolytic activity of CGTase. Mutation of Phe260 into a Leu, Ile, or Glu resulted in mutants with the highest hydrolysis activity ever reported for a CGTase. Furthermore, CGTase can be changed from a transglycosylase into a hydrolase by mutations in Phe260, a residue that is strongly conserved in CGTase proteins. The data presented provide new insights into the hydrolysis reaction catalyzed by CGTase and factors determining the transglycosylation versus hydrolysis specificity in the α -amylase family.

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